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14. ABSTRACT

Work has continued on the Top Down approach to genome minimization. We have used the Essential (E), Non-essential (N), and Impaired (I) gene categories to make steady progress with gene and gene cluster deletions. To date, we have removed approximately 234 kb from the *Mycoplasma mycoides* JCVI-syn1.0 genome. The resultant 844 kb genome is viable and grows with a normal growth rate.

The Bottom Up approach has also continued. New Tn5 gene disruption data allows a much more reliable classification of genes as E, I, or N. We have designed a new reduced genome design (RGD1) based on this data. Two initial segments from this 8 segment RGD1 design have been synthesized and are in testing for viability. We are also experimenting with an approach to construct RDG1 from PCR products amplified from syn1.0 genomic DNA. One 1/8th molecule is being synthesized by this PCR amplification method.

The effort to modularize the genome is in progress. A tRNA module has been constructed and is currently being sequenced prior to testing for the ability to replace the natural tRNA genes.

Preliminary work aimed at genome complementation has been conducted. A plasmid system for quickly adding back deleted genes will allow us to quickly examine which gene(s) within a deleted cluster might be causing growth defects or cell death. Experimental data using a two plasmid system suggests that development of a complementation system is possible.

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Construction of a Bacterial Cell that Contains Only the Set of Essential Genes Necessary to Impart Life

Report Title:	R&D Status Report (Quarterly)
Report Number:	HR0011-12-C-0063.5
Reporting Period:	May 17, 2013 to August 16, 2013
Contract No.:	HR0011-12-C-0063
Performing Organization:	J. Craig Venter Institute 9704 medical Center Drive Rockville, MD 20850 USA
Author of Report:	John Glass, Anthony Yee
Principal Investigators:	Hamilton Smith, Clyde Hutchison

Abstract

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Table of Contents:

Summary

The goal of the project is to create a cell that contains only the set of genes that are essential for life under ideal laboratory conditions. We are working to minimize *Mycoplasma mycoides* JCVI-syn1.0 (the synthetic version of *Mycoplasma mycoides* subsp *capri*) using two approaches:

- Top Down: remove genes and clusters of genes one (or a few) at a time, proceeding only if the reduced strain is viable, with a reasonable growth rate
    - We previously reported the results of a transposon study was conducted and allowed us to categorize genes as Essential (E), Non-essential (N), or Impaired (I)
        - This categorization scheme has been the basis of most of our subsequent work
    - The *M. mycoides* genome has been reduced to 844 kb using the Tandem Repeat Endonuclease Cleavage (TREC) strategy, with a doubling time of 68 minutes
    - By making gene cluster deletions in the 1/8th genome segments and assembling the segments into a genome, a 790 kb genome was made
        - The genome was viable, but had a doubling time of 82 minutes
- Bottom Up: design a reduced genome based on our best Tn5 gene disruption and deletion data (RGD1) , and synthesize it
    - Synthesis from oligonucleotides
        - The first two 1/8th genome molecules (sections 2 and 6) have been synthesized and sequence verified
        - Insertion of these sections into landing pad strains for viability testing is in progress
        - Transplantation experiments will be initiated by 23AUG13
        - Sections 3 and 5 will be synthesized next
    - Synthesis by PCR amplification and assembly via homologous recombination
        - Tests on segment 8 are in progress
        - Genes and clusters to be included in the reduced design have been amplified and transformed into yeast for assembly
        - Screening for correct assemblies is ongoing

The initial modularization experiments are progressing. A 5.3 kb tRNA module containing the 30 tRNA genes plus the necessary promoters and terminators was constructed and is in sequence verification.

Work has continued on the genome complementation front. We now have strong evidence that two plasmids can be transformed into the recipient cells.

Introduction

The goal of this research project is to build a minimal bacterial cell that contains only the genes that are required for life in ideal laboratory conditions. The pursuit of a minimized cell is critical to the advancement of biology, both as a pathway for understanding the basic requirements for replication and as a chassis for creating an optimized platform for any number of possible applications.

We previously reported that the *Mycoplasma mycoides* JCVI-syn1.0 genome was successfully reduced from 1078 kb to 779 kb; however, while the 779 kb genome was viable, the growth rate was far too slow to allow follow up experiments at an acceptable pace. Using the N, E, I gene categories, the genome has been reduced to 844 kb, but with a normal doubling time.

Synthesis of a newly designed reduced genome (RGD1, 539 kb) is ongoing at SGI. We expect that many of the $1/8^{th}$ genome segments from this design will prove to be viable. The first two $1/8^{th}$ molecules have been received and are being tested. Tests of a PCR-based method for synthesizing the same designed genome are in progress.

A preliminary tRNA module was designed and constructed.

Experiments on a plasmid-based genome complementation system are being conducted.

Methods, Assumptions and Procedures

TOP DOWN APPROACH

The plan here was to start with the full size 1078 kb *M. mycoides* JCVI-syn1.0 synthetic genome. The smallest viable genome to date was 779 kb, created using an 8-piece strategy to build genomes containing multiple deletions. However, the transplanted genome proved to grow very poorly in liquid culture. Using a less-reduced but faster growing strain as a starting point, we have successfully created a 790 kb strain, with an 82 min doubling time. Additional deletion selection has led to the design of a 690 kb strain.

We have continued to use the TREC strategy to make iterative deletions in the mycoplasma genome. Targeting the N category genes and clusters is proving to be effective (further discussed in the Results and Discussion section). We have made a series of strains that are progressively reduced with little to no reduction in growth rate.

BOTTOM UP APPROACH

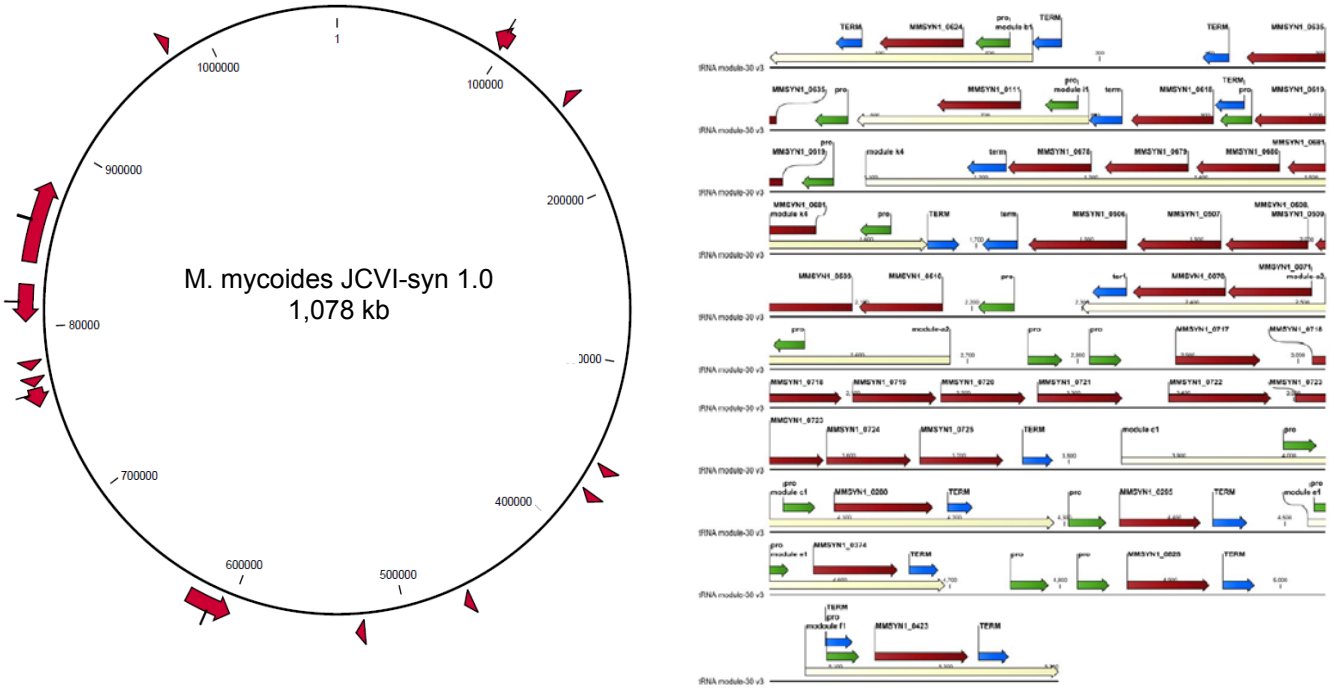
Synthesis from oligonucleotides: A new eight piece genome design was completed using the N, E, I gene classification system (RGD1, 539 kb). The designs were provided to SGI, and the first two segments (2 and 6) have been sequence verified and returned. Each segment is being inserted into a 7/8 normal landing pad strain for testing. This will be completed shortly. The resultant genomes will then be transplanted to test for viability.

Synthesis by PCR amplification of targeted regions: Segment 8 contains 11 regions that are retained in the design, ranging from 200 bp to 27 kb. Regions smaller than 10 kb were PCR amplified as a single fragment while larger regions were split into multiple fragments. Primers were designed to produce fragments that overlap by 60-70 bp. The assembly of the reduced segment 8 (14 fragment in total and a vector) was performed in yeast using spheroplast transformation. Currently colonies are being screen to identify correct assemblies. The next step will be to combine the reduced segment 8 with the 7/8 genome (which is maintained in yeast) and test for viability by performing transplantation experiments.

MODULARIZATION

To test gene modularization, we have organized the 30 tRNA genes of *M. mycoides* into a single contiguous module. The module contains the coding regions, as well as the promoters and terminators needed for regulation. The module was sent for sequence verification. Any errors must be corrected before we can test the module. The tRNA genes are naturally distributed around the genome in 13 loci, which we plan to disrupt using the Green Monster method.

Figure 1



(a) Natural distribution of tRNA genes in M. mycoides. *The tRNA gene clusters have been enlarged in Fig.1(a) to show the direction of transcription. The M. mycoides JCVI-syn1.0 genome has 8 single tRNA genes and 5 clusters of 2 to 9 genes, for a total of 30.*

(b) tRNA module design. *The 30 tRNA genes have been relocated into a single module. (Green arrows represent promoters. Red arrows show tRNA genes. Blue arrows are terminators.)*

GENOME COMPLIMENTATION

We have begun initial experiments aimed to enable genetic complementation to restore desirable phenotypes to deletion mutants. A plasmid system capable of replication in both *M. mycoides* and in the genome transplantation recipient cell, *M. capricolum* would be a powerful tool to help de-convolute growth-retarding synthetic effects. Previous attempts to transform a plasmid into a recipient cell, then perform transplantation and simultaneously select for the plasmid and the transplanted genome have not been successful. Experiments to add a second plasmid to *M. capricolum* cells that already harbor one plasmid have required a high amount of the second plasmid. This suggests that we have a path forward to develop the complementation system. By increasing the amount of the donor genome, we hope to achieve transplantation and maintenance of the initial plasmid.

Results and Discussion

TOP DOWN APPROACH

Iterative deletions using the TREC based approach are making steady progress toward a minimal genome. A table outlining the progress to date is shown below. Strain D10 has been designed, but testing for viability has not been completed.

Strains	DT(min)	Genome Size (bp)	# of Genes Deleted
syn1.0	≈70	1,078,809	0
syn1.0D6 RE	≈70	1,062,183	17
DISs	≈70	1,048,690	31
D1	≈70	979,083	68
D2	≈70	969,069	74
D3	≈70	944,159	90
D4	≈70	931,710	97
D5	≈70	923,647	102
D6	68	908,931	108
D7	72.2	877,942	135
D8	70	866,271	145
D9	68	844,265	163
D10	TBD	828,901	181

MODULARIZATION

We have moved ahead with synthesis of a tRNA module. Construction was difficult because the complex secondary structures of the tRNAs interfered with the synthesis process. The module is complete and has been sent for sequence verification.

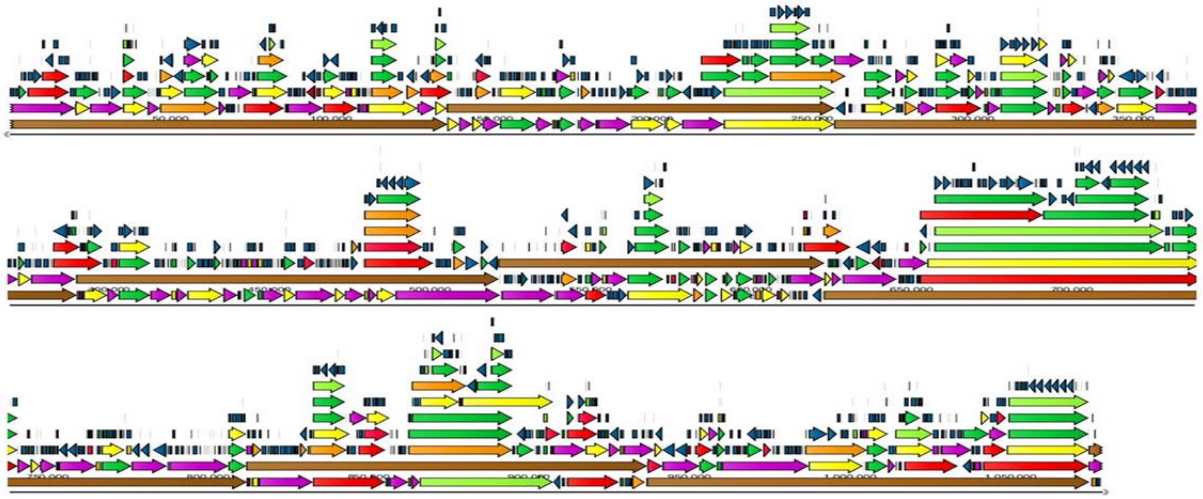
BOTTOM UP APPROACH

A new genome design (RGD1) was completed using the N, E, I classification system. The design essentially involves removing all of the N (non-essential) genes and then checking to see that we have not disturbed the promoters and terminators necessary for expression of the remaining E (essential), and I (impaired growth if deleted) genes. Segments 2 and 6 have now been synthesized sequence verified. These will be tested for viability next week in Rockville. Segments 3 and 5 will be synthesized next.

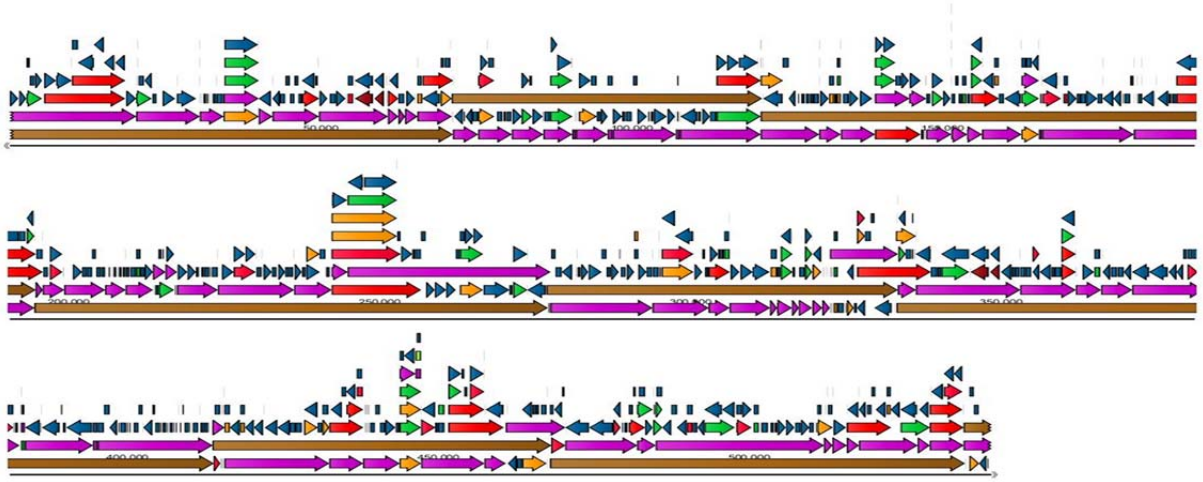
Fig.2, below, shows the contrast between (a) the *M. mycoides* JCVI-syn1.0 genome and (b) the new genome design.

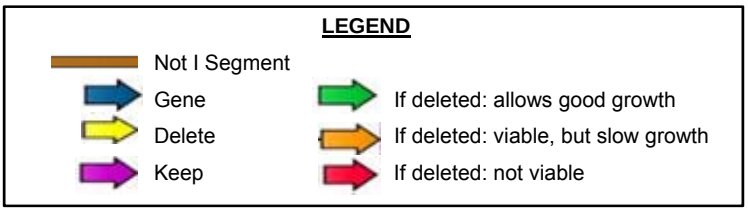
Figure 2

(a)



(b)





(a) The 1,078 kb M. mycoides JCVI-syn1.0 genome, showing the genes retained in the new genome design (purple arrows)*. Note that genes in the new design are interspersed around the genome, comingled with genes that were removed from the design;* ***(b) The new design of a minimized, 539 kb M. mycoides genome****. Contrast the spacing between the retained genes (purple) with the spacing in (a).*

If a lethal deletion (red) falls entirely within a region deleted in the design (yellow) then there is an inconsistency between the design and the deletion data. Where the data from different deletions disagrees, green trumps red because failure to get a transplant is a negative result that could result from some problem with that particular transplantation reaction.

The table below shows the length of each 1/8th genome Not I segment in the *M. Mycoides* JCVI-syn1.0 genome, and the newly designed RGD1 genome. The new design represents a 50% reduction in total genome length.

Not I Fragment #	*M. Mycoides* JCVI-syn1.0 Length (bp)	RGD1 Designed Length (bp)	(RGD1)/(*M. Mycoides* JCVI-syn1.0)
1	140,739	75,732	0.54
2	120,912	49,888	0.41
3	133,208	73,958	0.56
4	131,623	82,531	0.63
5	101,708	56,501	0.56
6	189,357	80,747	0.43
7	124,976	54,482	0.44
8	137,887	66,717	0.48
Total	1,080,410	540,566	
Overlaps	-1,601	-1,601	
Genome Length	1,078,809	538,955	0.50

Conclusions

Tasks from the Statement of Work for Year 1:

Task 1: **Complete a detailed global Tn5 transposon mutagenesis insertion map.**
The Tn5 transposon insertion map was submitted with the initial quarterly report.

Due: Month 6; Status - complete

Task 2: **Delete up to 27 large gene clusters**
We have reduced the genome size of M. mycoides JCVI-syn1.0 from 1079 kb to 779 kb through the deletion of some 30 clusters, representing a ~30% reduction. We are moving forward with an 848 kb genome because it grows at a higher rate than the smaller 779 kb version.

Due: Month 12; Status – complete

Task 3: **Construct a preliminary modular map of the genome**
The design of a modular map of the genome is complete was presented in the May 2012 quarterly report

Due: Month 12; Status – complete

Task 4: **Make new transposon insertion map. Identify non-essential small 2-4 gene clusters. Delete small clusters.**
A new transposon study was performed. We continue to work toward a minimal cell chassis using multiple simultaneous approaches.

Due: Month 18 (November 2013); Status – in progress

Planned Activities for the Next Reporting Period

1. Continue the Top Down minimization of our synthetic genome (near term) and conduct a new transposon study on the minimized genome (within approximately 4-6 months)
2. We will continue with synthesis and testing of the 8 segments of the RGD1 genome as part of our Bottom Up strategy.
3. Continue with verification and testing of the tRNA gene module.
4. Continue development of genome complementation strategies

Program Financial Status

	Planned Expend	Actual Expend (Cumulative to Date)	% Budget Completion	At Completion	Latest Revised Estimate	Remarks
Task 1	$305,646	$305,646	100%	$305,646	$305,646	Completed
Task 2	$826,256	$798,351	97%	$798,351	$826,256	Completed
Task 3	$43,487	$43,487	100%	$43,487	$43,487	Completed
Task 4	$634,981	$151,514	24%	N/A	$634,981	N/A
Cumulative	$1,810,370	$1,298,998	72%	N/A	$1,810,370	N/A

There is no management reserve or unallocated resources. The financial data presented is current through June 30, 2013.

Based on the currently authorized work:

- Is current funding sufficient for the current fiscal year? Yes
- What is the next fiscal year funding requirement at current anticipated levels? $1,214,151.00
- Have you included in the report narrative any explanation of the above data and are they cross-referenced? Not applicable; current funding is sufficient for the current fiscal year.